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Molecular analysis of a new cytoplasmic male sterile genotype in sunflower

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Mitochondrial DNA from 1 fertile and 6 cytoplasmic male sterile (CMS) sunflower genotypes was studied. The CMS genotypes had been obtained either by specific crosses between different *Helianthus* species or by mutagenesis. CMS-associated restriction fragment length polymorphisms (RFLPs) were found in the vicinity of the *atpA* locus, generated by various restriction enzymes. The organization of the mitochondrial genes *26S rRNA*, *18S+5S rRNA* and *coxII* was investigated by Southern blot analysis. These genes have similar structures in fertile and all studied sterile sources. Using the *atpA* probe, 5 from the 6 investigated CMS genotypes showed identical hybridization patterns to the *Petiolaris* CMS line, which is used in all commercial sunflower hybrids. Only 1 cytoplasm derived from an open pollination of *Helianthus annuus* ssp. *texanus*, known as ANT_1, contained a unique mitochondrial DNA fragment, which is distinguishable from the fertile and sterile *Petiolaris* genotypes and from all investigated CMS genotypes. Male fertility restoration and male sterility maintenance of the ANT_1 line are different from the *Petiolaris* CMS system, which is a confirmation that a novel CMS genotype in sunflower has been identified.

Sunflower; Mitochondrial genome; Cytoplasmic male sterility; RFLP; *atpA* locus

1. INTRODUCTION

Cytoplasmic male sterility (CMS) is a maternally inherited trait in higher plants that results in the inability of the mature plant to produce functional pollen, but it does not affect female fertility [1]. In sunflower a CMS genotype was obtained from an interspecific cross between *Helianthus petiolaris* and *H. annuus* which was first described by Leclercq [2]. The subsequent identification of male fertile lines containing specific dominant nuclear genes which restore pollen fertility [3–5] resulted in a rapid production and cultivation of sunflower hybrids.

In a number of cases analysed, the CMS phenotype is suggested to originate from mutations in the mitochondrial genome of the male fertile progenitors as a result of intra- or intermolecular recombination events. The mitochondrial genome rearrangements have generated chimaeric mtDNA sequences which in some cases result in generation of novel mitochondrial genes or lead to a modification of existing genes [6]. These chimaeric genes are expressed as novel or modified polypeptides which, in an unknown fashion, are related to a failure in mitochondrial function during development of the pollen.

Our current knowledge about the molecular basis of CMS mainly comes from studies performed in maize and petunia. The chimaeric mitochondrial gene *T-urf 13*, composed primarily of sequences derived from the *26S rRNA* and the *atp6* gene, is unique for maize with Texas male sterile cytoplasm and codes for a 13-kDa polypeptide [7,8]. In reversion of CMS-T maize to fertility *T-urf 13* is deleted or truncated through recombination [9–12]. Two dominant nuclear genes, Rf_1 and Rf_2, restore pollen fertility and reduce the abundance of the 13-kDa polypeptide [7]. In petunia the CMS phenotype was also shown to be associated with a specific DNA segment of the mitochondrial genome [13,14]. The DNA sequence and transcript pattern of the CMS-associated region, *pcf-S*, were determined [15,16]. A 25-kDa protein associated with CMS in petunia was identified. One nuclear gene Rf is sufficient to confer fertility and reduce the abundance of this 25-kDa *pcf-S* protein [17].

The use of only 1 sunflower CMS source on a large scale may lead to a reduction of the genetic variability of the breeding material and to genetic vulnerability to diseases. A convincing example of the latter is the maize Texas CMS which is susceptible to Southern corn leaf blight [18]. Obviously it is important to increase the cytoplasmic genetic diversity in crop plants by identi-

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fying or creating new sources of male sterility and also to investigate the molecular, biochemical and physiological basis of CMS. Little is known about the molecular determination of CMS in sunflower. In the restriction map of the mitochondrial DNA of sunflower an area of 17 kb, including the *atpA* gene, is different in the *Petiolaris* CMS line compared to its fertile analogues [19]. In this paper we present our study of 6 new sunflower CMS genotypes. These genotypes were obtained from intra- or interspecific crosses of *Helianthus* species or mutagenesis of sunflower cultivars, and have been characterized for further utilization in breeding programmes.

2. MATERIALS AND METHODS

2.1. *Plant material*

Plants were grown in the field and leaves, used for DNA isolation, were harvested before flowering. The origins of the different cytoplasmic male sterile genotypes are described in Table I.

2.2. *Isolation of mitochondrial DNA*

50 g of leaf material was homogenized in 250 ml ice-cold buffer comprising 0.44 M mannitol, 50 mM Tris-HCl, 3 mM Na_2EDTA, 0.2% Polyclar, 0.1% bovine serum albumin, 10 mM 2-mercaptoethanol, pH 8.0. The ruptured cells were filtered through cheesecloth and miracloth. The debris and crude chloroplast fraction were collected at 3,000 × *g* for 15 min, followed by a centrifugation at 18,000 × *g* for 20 min to sediment the mitochondria. Mitochondrial pellets were resuspended in 1 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and lysed with 100 μl 10% SDS and 100 μl 10% Sarkosyl during an incubation of 20 min at 65°C. For protein precipitation 100 μl ice-cold 5 M potassium acetate was added, followed by an incubation for 20 min at 4°C. The precipitated protein complexes were removed by centrifugation at 12,000 × *g* for 10 min and the supernatants were collected by filtration through cheesecloth for further purification by phenol-chloroform extractions and ethanol precipitations. The nucleic acid pellets were dissolved in 0.4 ml sterile water and treated with RNAse 10 mg/ml for 30 min at 37°C. After 2 subsequent phenol-chloroform extractions and a final ethanol precipitation the mtDNA was resuspended in 100 μl TE.

Table I

Origins of cytoplasmic male sterile genotypes in sunflower

Code	Fertility	Origins	References
HA_{89}	F	*H. annuus*	Leroy et al. [27]
CMS_{89}	S	*H. petiolaris* X *H. annuus*	Leroy et al. [27]
CMS_1	S	*H. argophyllus* X *H. annuus*	Christov [28]
CMS_2	S	γ irradiation of cultivar Hemus	Christov (unpublished results)
CMS_3	S	*H. annuus* ssp. *texanus*, open pollination	Vranceanu et al. [26]
CMS_4	S	sonication of cultivar Peredovic	Christov (unpublished results)
CMS_5	S	*H. scaberimus* X *H. annuus*	Bohorova (unpublished results)
CMS_6	S	*H. annuus* X *H. hirsutus*	Bohorova et al. [29]

F, fertile; S, sterile

2.3. *DNA Analysis*

About 2 μg mtDNA was digested with 20 U of restriction enzyme (Bethesda Research Laboratories), fractionated by electrophoresis on a 1% agarose gel and blotted onto Hybond N^+ by vacuum blotting (LKB). Hybridization with random priming labeled probes was carried out in 10% dextran sulphate, 1 M NaCl, 1% SDS and 200 μg/ml denatured herring sperm DNA at 60°C. After washing down to 0.1 SSC at 60°C, blots were autoradiographed using Kodak X-Omat AR films.

The probes used in this analysis have been provided by Dr. Toro Terachi, Kyoto Sangyo University, Japan (personal communication). The following mitochondrial probes were used: *atpA* (1.5-kb *Hin*dIII–*Eco*RI fragment, containing the coding region of subunit A of the ATPase gene of *Pisum sativum*); *coxII* (1.9-kb *Eco*RI fragment, containing the coding region of the cytochrome *c* oxidase subunit II of *Pisum sativum*); *18 S + 5 S rRNA* (a 3.2-kb *Bam*HI–*Sal*I fragment which contains the 5′ upstream region of the 18 S and 5 S rRNA genes and also the tRNA(fMet) gene from *Triticum aestivum* [20]); and *26S rRNA* (a 5.2-kb *Bam*HI–*Sal*I fragment from *Triticum aestivum* which contains part of the 5′ upstream region of the 26 S rRNA gene [21]).

3. RESULTS

Southern blot analyses were performed to investigate the mitochondrial genomes of several new sunflower CMS sources. Digested and electrophoretically separated mtDNA was blotted to nylon membranes and hybridized with different clones of mitochondrial genes. The results show that the *atpA* probe hybridized to DNA fragments of different sizes in fertile- (F) as compared to sterile- (S) and investigated new CMS genotypes, when digested with the restriction endonucleases *Bst*EII and *Sal*I (Fig. 1). The *atpA* probe hybridized to a 13.9-kb *Bst*EII fragment and a 4.4-kb *Sal*I fragment from the F line and to a 5.8-kb *Bst*EII and 7.0-kb *Sal*I fragment from the S line. Some weak hybridization can also be observed in addition to the main bands both in the F, S and CMS lines. This probably is due to short repeated sequences as has also previously been reported by Köhler et al. [22]. It was demonstrated that the hybridization pattern of five out of six investigated CMS sources is identical to the *Petiolaris* sterile genotype, despite the fact that they were developed from different crosses between *Helianthus* species or by mutagenesis of sunflower cultivars and manifest different morphological and physiological characteristics.

One of the investigated lines, CMS_3, showed a different restriction fragment length polymorphism (RFLP) distinguishing both F- and S lines. The *atpA* probe hybridized to a 9.2-kb *Bst*EII fragment and a 1.5-kb *Sal*I fragment of CMS_3 mtDNA indicated as NS (new sterile). Different hybridization patterns were also found when the *atpA* probe was hybridized to mtDNA from F-, S- and NS lines after digestion with the enzymes *Hin*dIII, *Bgl*I, *Pst*I, *Bst*EII/*Bgl*I, *Bst*EII/*Sal*I and *Hin*dIII/*Sal*I. Fig. 2 presents the RFLPs between F-, S- and NS lines obtained after double digestions with *Bst*EII/*Bgl*I-*Bst*EII/*Sal*I and using the *atpA* probe. The results from Southern blot analysis have been used to

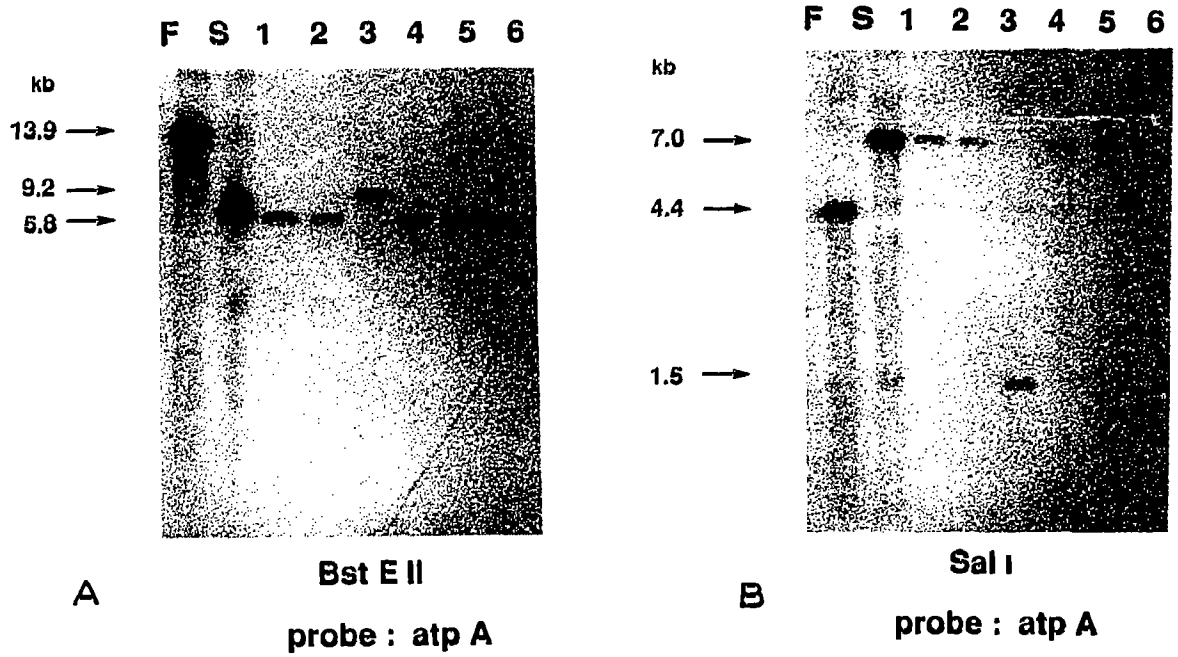

Fig. 1. Southern analysis of mtDNA from fertile and CMS sunflower lines. (A) Southern hybridization pattern of *Bst*EII-digested mtDNA from the fertile (F) line HA_{89}, the sterile (S) line CMS_{89} and 6 new CMS sunflower sources (described in Materials and Methods and Table I) hybridized with a 1.5-kb fragment containing the coding region of the *atpA* gene. Lanes 1–6 correspond to CMS_1–CMS_6. (B) Southern hybridization pattern of *Sal*I-digested mtDNA from F-, S- and new CMS sunflower sources hybridized to the *atpA* gene probe.

create restriction maps of the *atpA* area in mtDNAs from fertile HA_{89}, sterile CMS_{89} and new sterile CMS_3 sunflower lines (Fig. 3). In this figure it is shown that to the left of the *Sal*I site in the *atpA* gene the mtDNA organization of the F-, S- and NS lines is colinear. Differences in the mtDNA organization between F-, S- and NS lines are observed to the right of the *Sal*I site. These results emphasize the differences in genome organization between F-, S- and NS lines and exclude the probability of a point mutation in the mitochondrial genome of the NS sunflower line.

In order to further analyze the mtDNA organization in F-, S- and the described CMS lines, and to clarify whether rearrangements took place elsewhere in the mitochondrial genomes, we carried out Southern hybridization analyses with cloned mitochondrial genes from *P. sativum* and *T. aestivum* as heterologous probes. The genes used were *coxII*, *26S rRNA*, *18S + 5S rRNA*. The results, shown in Fig. 4, indicate that *coxII* hybridized to the mtDNA from F-, S- and (NS) lines double-digested with *Bst*EII/*Bgl*I-*Bst*EII/*Sac*I and *Bst*EII/*Sal*I without any differences among the various lines. No differences were detected either in hybridization patterns using other enzymes like *Hin*dIII, *Bgl*I, *Pst*I and the other mitochondrial probes, including *26S rRNA*, *18S+5S rRNA* and *coxII*. It is obvious that these regions have a similar structure in fertile and all sterile sources and probably are not involved in recombination events associated with CMS in sunflower.

4. DISCUSSION

At present all commercial sunflower hybrids contain the *Petiolaris* CMS which was found by Leclercq [2]. In order to be able to introduce cytoplasmic diversity into sunflower we analyzed in this study the high molecular weight mtDNA of possible new types of sunflower CMS. Studies were conducted on 6 CMS genotypes and a near-isogenic male sterile and male fertile sunflower line in Southern blot analyses using 10 restriction endonucleases and 5 mitochondrial genes. The *atpA* probe distinguished between the CMS S- and F-type in the hybridization experiments with all tested restriction enzymes. Out of the 6 investigated CMS genotypes 5 showed identical hybridization patterns indistinguishable from the well known *Petiolaris* CMS. No specific restorer sunflower lines have been found for the different CMS genotypes. However, some *Petiolaris* CMS maintainers partially restore male fertility of these sunflower CMS genotypes.

During preparation of this manuscript Crouzillat et al. [23] reported the genetic analysis and molecular basis of 15 sunflower CMS sources which have different origins than the genotypes included in our studies. In agreement with our results they found identical hybridization patterns with the *atpA* gene and 3 restriction enzymes between *Petiolaris* CMS and a CMS line originating from *H. anomalus*. Although this CMS line shows additional RFLPs detected by *coxII*, *cob* and

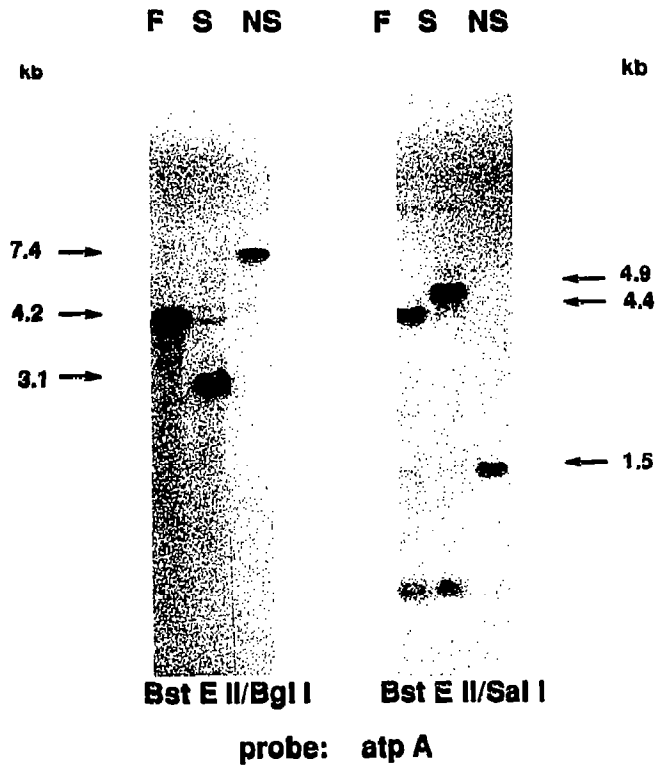

Fig. 2. Southern blot analysis of mtDNA isolated from a fertile HA_{89} (F), a sterile CMS_{89} (S) and CMS_3 (NS) line, obtained from an open pollination of *H. annuus* ssp. *texanus*. The mtDNA, double-digested with *Bst*EII/*Bgl*I and *Bst*EII/*Sal*I was hybridized with a random priming labeled *atpA* probe. The arrows indicate the CMS-specific polymorphisms between F-, S- and NS lines.

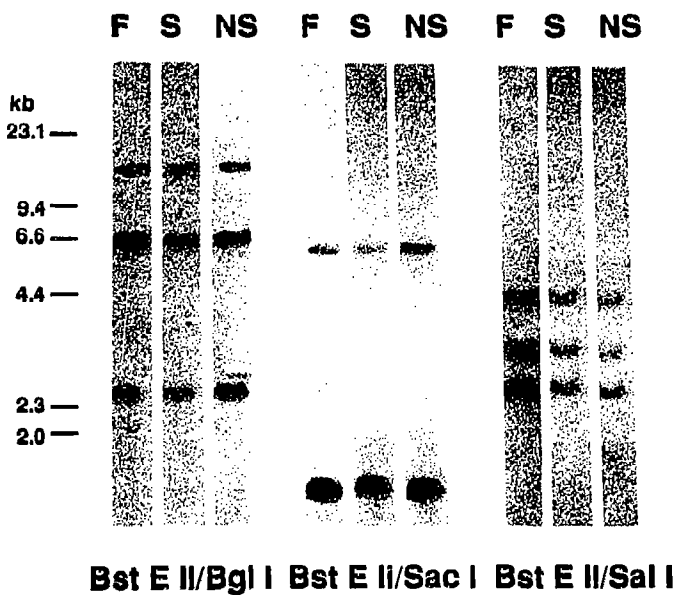

Fig. 4. Hybridization of mitochondrial gene *coxII* to restriction fragments of sunflower mtDNA from fertile (F), sterile (S) and new sterile (NS) lines, generated by double-digestion with *Bst*EII/*Bgl*I, *Bst*EII/*Sac*I and *Bst*EII/*Sal*I. The hybridization pattern shows no differences between F-, S- and NS lines.

atp9, these results lead to the suggestion that probably, by being affected in the *atpA* area the different sunflower CMS genotypes are related.

The organization of the mitochondrial genomes of the *Petiolaris* CMS genotypes and all studied new CMS lines regarding the 4 other genes which have been examined (*coxII*, *26S rRNA*, *18S+5S rRNA*) is identical to the F-type. According to Crouzillat et al. [23] the genes *26S rRNA*, *18S+5S rRNA* show less variability than those coding for ATPases, but they could still find RFLP differences in 3 groups of CMS cytotypes. Using 3 enzymes and 12 probes per genotype they found 20% RFLPs (using the *26S rRNA* probe) and 33.4% RFLPs (using the *18S + 5S rRNA* probe) which differ from the F genotype [23]. In our study we tested 40 different enzyme/probe combinations (except *atpA*) for each CMS genotype and could not find any RFLP. No differences, not only for the *26S rRNA* and *18S + 5S rRNA* but also for the *coxII* gene, have been found, which is an indication that these loci are not involved

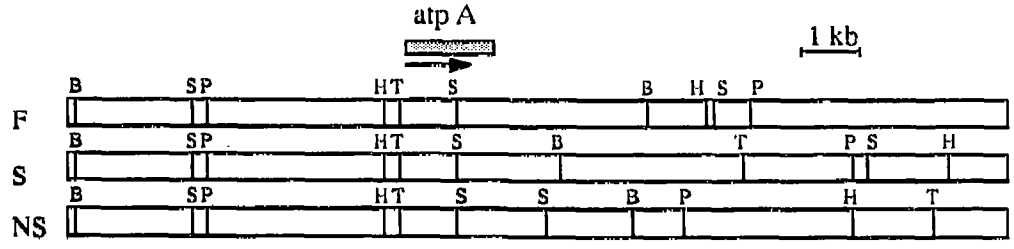

Fig. 3. Restriction maps of the surrounding regions of the *atpA* locus in fertile (F), sterile (S) and CMS_3 (NS) lines. Restriction sites shown: (T) *Bst*EII; (B) *Bgl*I; (S) *Sal*I; (H) *Hin*dIII; and (P) *Pst*I. The maps have been created on the basis of Southern blot analysis and [19]. The arrow under the *atpA* gene indicates the direction of transcription according to [22].

in events associated with CMS. This does not exclude the possibilities of other RFLP differences elsewhere in the mitochondrial genomes, which could be hypothesized as additional deficiencies responsible for CMS. However, recent publications show that in the commonly used CMS sunflower genotype there is a correlation between CMS and co-transcription of a new open reading frame with the *atpA* gene [22,24]. Probably the translation product of this open reading frame is a 16-kDa polypeptide which is suggested to play a role in the CMS phenotype [25]. This also points to the area of the *atpA* locus to be involved in CMS in sunflower.

One of the investigated lines, CMS_3, obtained from an open pollination of *H. annuus* ssp. *texanus* and known as ANT_1 [26], showed a different RFLP distinguishing this NS line from both F- and S genotypes and all new investigated sunflower CMS lines. This NS line was characterized by a complete anther and pollen atrophy and has proven to be stable under various environmental conditions. Classical restorers commonly used in the *Petiolaris* CMS system do not restore fertility of this NS line and up until now no sunflower lines have been found that restore the fertility of the NS line ([26], P. Petrov, unpublished results). These results agree with the molecular characterization and suggest the existence of a novel type of CMS in sunflower, originating from rearrangements in the vicinity of the *atpA* gene. The *atpA* probe in the investigations of Crouzillat et al. [23] detected 8 cytotypes among 15 sunflower cytoplasms; apparently the *atpA* gene is involved somehow in many sunflower CMSs. In order to understand the molecular basis of CMS in sunflower future experiments will concentrate on differences in organisation and expression of genes in the area of the *atpA* locus between F-, S- and NS genotypes.

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REFERENCES

[1] Hanson, M.R. and Conde, M.F. (1985) Int. Rev. Cytol. 94, 213–267.
[2] Leclercq, P. (1969) Ann. Amél. Plantes 19, 99–106.
[3] Leclercq, P. (1971) Ann. Amél. Plantes 21, 45–54.
[4] Vranceanu, A.V. and Stoenescu, F.M. (1971) Euphytica 20, 536–541.
[5] Vranceanu, A.V. and Stoenescu, F.M. (1978) Euphytica 27, 617–627.
[6] Levings III, C.S. and Brown, G.G. (1989) Cell 56, 171–179.
[7] Dewey, R.E., Levings III, C.S. and Timothy, D.H. (1986) Cell 44, 439–449.
[8] Dewey, R.E., Timothy, D.H. and Levings III, C.S. (1987) Proc. Natl. Acad. Sci. USA 84, 5374–5378.
[9] Fauron, C.M.R., Abbott, A.G., Brettell, R.I. and Gesteland, R.F. (1987) Curr. Genet. 11, 339–346.
[10] Fauron, C.M.R., Havlik, M. and Brettell, R.I.S. (1990) Genetics 124, 423–428.
[11] Rottmann, W.H., Brears, T., Hodge, T.P. and Lonsdale, D.M. (1987) EMBO J. 6, 1541–1546.
[12] Wise, R.P., Pring, D.R. and Gengenbach, B.G. (1987) Proc. Natl. Acad. Sci. USA 84, 2858–2862.
[13] Boeshore, M.L., Hanson, M.R. and Izhar, S. (1985) Plant Mol. Biol. 4, 125–132.
[14] Hanson, M.R., Pruitt, K.D. and Nivison, H.T. (1989) Oxford Survey Plant Mol. Cell Biol.
[15] Pruitt, K.D. and Hanson, M.R. (1989) Curr. Genet. 16, 281–291.
[16] Rasmussen, J. and Hanson, M.R. (1989) Mol. Gen. Genet. 215, 332–336.
[17] Nivison, H.T. and Hanson, M.R. (1989) Plant Cell 1, 1121–1130.
[18] Tatum, L.A. (1971) Science 171, 1113–1116.
[19] Siculella, L. and Palmer, J. (1988) Nucleic Acids Res. 16, 3787–3799.
[20] Falconet, D., Lejeune, B., Quetier, F. and Gray, M. (1984) EMBO J. 3, 297–302.
[21] Falconet, D., Delorme, S., Lejeune, B., Sevignac, M., Delcher, E., Bazetoux, S. and Quetier, F. (1985) Curr. Genet. 9, 169–174.
[22] Köhler, R.H., Horn, R., Lössl, A. and Zetsche, K. (1991) Mol. Gen. Genet. 227, 369–376.
[23] Crouzillat, D., de la Canal, L., Perrault, A., Ledoigt, G., Vear, F. and Serieys, H. (1991) Plant Mol. Biol. 16, 415–426.
[24] Laver, H.K., Reynolds, S.J., Moneger, F. and Leaver, C.J. (1991) Plant J. 2, 185–193.
[25] Horn, R., Köhler, R.H. and Zetsche, K. (1991) Plant Mol. Biol. 17, 19–27.
[26] Vranceanu, A.V., Iuoras, M. and Stoenescu, F.M. (1986) HELIA 9, 21–24.
[27] Leroy, P., Bazetoux, S., Quetier, F., Delbut, J. and Berville, A. (1985) Curr. Genet. 9, 245–251.
[28] Christov, M. (1990) PhD Thesis, Agricultural Academy of Science, Bulgaria.
[29] Bohorova, N., Spirova, M. and Atanassov, A. (1987) Genet. Sel. 20, 204–210.